A Glio-Protective Role of mir-263a by Tuning Sensitivity to Glutamate

Aw, Sherry Shiying; Lim, Isaac Kok Hwee; Tang, Melissa Xue Mei; Cohen, Stephen Michael

Published in:
Cell Reports

DOI:
10.1016/j.celrep.2017.05.010

Publication date:
2017

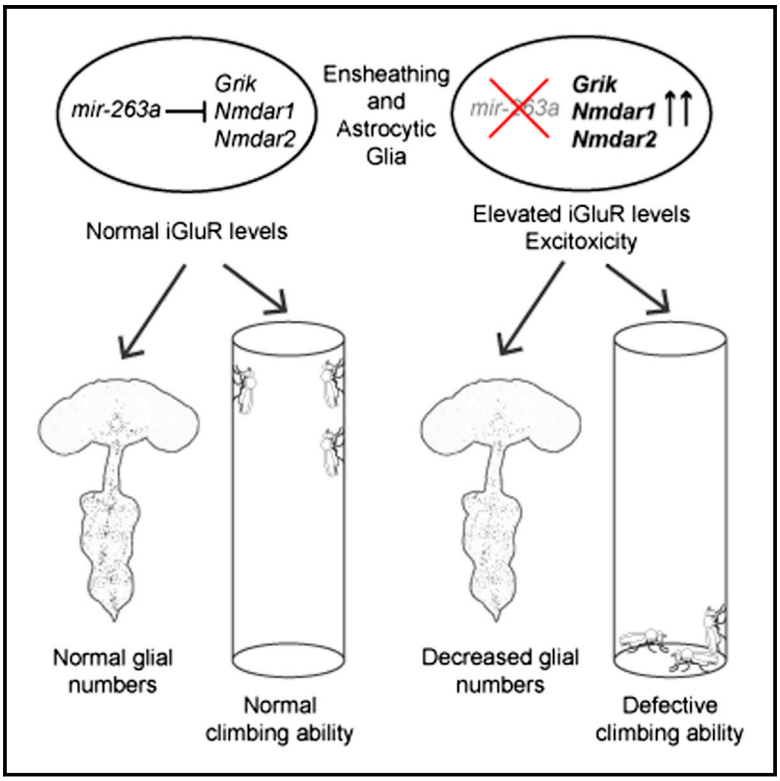
Document version
Publisher's PDF, also known as Version of record

Article

Cell Reports

A Glio-Protective Role of *mir-263a* by Tuning Sensitivity to Glutamate

Graphical Abstract



Highlights

- *mir-263a* mutants exhibit death of a subset of astrocyte-like and ensheathing glia
- These defects are associated with severe movement defects
- *mir-263a* regulates glutamate receptor levels to protect glia from excitotoxicity

Authors

Sherry Shiying Aw, Isaac Kok Hwee Lim, Melissa Xue Mei Tang, Stephen Michael Cohen

Correspondence

syaw@imcb.a-star.edu.sg (S.S.A.), scohen@sund.ku.dk (S.M.C.)

In Brief

Excessive glutamatergic signaling can cause neurodegeneration. Aw et al. report that *Drosophila mir-263a* limits glutamate receptor levels in a subset of glia, protecting them from excitotoxicity. *mir-263a* mutants exhibit severe movement defects. This study reveals a mechanism by which glia protect themselves from excess glutamate signaling to maintain CNS health.

Aw et al., 2017, Cell Reports *19*, 1783–1793
May 30, 2017
http://dx.doi.org/10.1016/j.celrep.2017.05.010

CellPress

Cell Reports
Article

A Glio-Protective Role of *mir-263a* by Tuning Sensitivity to Glutamate

Sherry Shiying Aw,[1,3,*] Isaac Kok Hwee Lim,[1] Melissa Xue Mei Tang,[1] and Stephen Michael Cohen[1,2,*]
[1]Institute of Molecular and Cell Biology, 61 Biopolis Drive, Singapore 138673, Singapore
[2]Present address: Department of Cellular and Molecular Medicine, University of Copenhagen, Blegdamsvej 3, Copenhagen 2200N, Denmark
[3]Lead Contact
*Correspondence: syaw@imcb.a-star.edu.sg (S.S.A.), scohen@sund.ku.dk (S.M.C.)
http://dx.doi.org/10.1016/j.celrep.2017.05.010

SUMMARY

Glutamate is a ubiquitous neurotransmitter, mediating information flow between neurons. Defects in the regulation of glutamatergic transmission can result in glutamate toxicity, which is associated with neurodegeneration. Interestingly, glutamate receptors are expressed in glia, but little is known about their function, and the effects of their misregulation, in these non-neuronal cells. Here, we report a glio-protective role for *Drosophila mir-263a* mediated by its regulation of glutamate receptor levels in glia. *mir-263a* mutants exhibit a pronounced movement defect due to aberrant overexpression of *CG5621/Grik*, *Nmdar1*, and *Nmdar2*. *mir-263a* mutants exhibit excitotoxic death of a subset of astrocyte-like and ensheathing glia in the CNS. Glial-specific normalization of glutamate receptor levels restores cell numbers and suppresses the movement defect. Therefore, microRNA-mediated regulation of glutamate receptor levels protects glia from excitotoxicity, ensuring CNS health. Chronic low-level glutamate receptor overexpression due to mutations affecting microRNA (miRNA) regulation might contribute to glial dysfunction and CNS impairment.

INTRODUCTION

Glutamate is an abundant neurotransmitter in vertebrates and invertebrates. Glutamatergic neurotransmission is mediated by ionotropic and metabotropic glutamate receptors. Ionotropic receptors of the AMPA, NMDA, and kainate types are cation-permeable, membrane-bound ion channels, while metabotropic receptors are G protein coupled and function through a secondary messenger. Glutamatergic signaling is critical for normal synaptic and circuit development, as well as for mature CNS functions, including cognition, learning, and memory. Reduced receptor function retards synaptic development and alters normal neuronal function (reviewed by van Zundert et al., 2004; Zhang and Poo, 2001). Excessive receptor activation, on the other hand, can lead to excitotoxic cell death, which is associated with many CNS pathologies (Choi, 1988; Zipfel et al., 2000). The subunit composition of individual receptors confers different ligand binding and channel properties; consequently, glutamate receptor expression, assembly, trafficking, and activity are highly regulated (Sanz-Clemente et al., 2013).

In addition to cell-intrinsic regulation of glutamate responsiveness in neurons, clearance of released neurotransmitters from the synaptic cleft is essential for synaptic function. Astrocytic glia take up extracellular glutamate for recycling via excitatory amino acid transporters (EAATs) (Anderson and Swanson, 2000). Sustained activation of neuronal glutamate receptors due to defects in glutamate clearance can result in excitotoxicity (Rothstein et al., 1996). Evidence is emerging that glutamatergic signaling is also important in glia. Astrocytes and oligodendrocytes (which produce the myelin sheaths that wrap neuronal projections) express all the major classes of glutamate receptors (Porter and McCarthy, 1997; Verkhratsky and Steinhäuser, 2000) and are susceptible to injury from excess exposure to glutamate in vitro (McDonald et al., 1998; David et al., 1996; Chen et al., 2000). Glia can interact with neurons in vitro by sending and receiving glutamatergic signals (Parpura et al., 1994; Cornell-Bell et al., 1990; Lalo et al., 2006). Interestingly, glial glutamate receptors may differ in composition and properties from neuronal glutamate receptors (Palygin et al., 2011). The importance of glial glutamate receptor function in normal brain physiology is only beginning to be understood (Kolodziejczyk et al., 2010; Saab et al., 2012).

In this report we present evidence for microRNA-mediated regulation of glutamate receptors in glia. Neuroprotective roles have previously been documented for neuronally expressed microRNAs (Kim et al., 2007; Karres et al., 2007; Liu et al., 2012; Gehrke et al., 2010). Neuronal *mir-181* and *mir-223* regulate glutamate receptors, thereby affecting postsynaptic neurotransmitter responsiveness (Saba et al., 2012; Harraz et al., 2012). *mir-223*-mediated repression of glutamate receptors in hippocampal neurons has been shown protect against excitotoxic cell death (Harraz et al., 2012). Presynaptic activity of *mir-1000/137* has also been shown to be neuroprotective via regulation of vesicular glutamate transporter expression (Verma et al., 2015). Blocking overall microRNA (miRNA) biogenesis through deletion of Dicer from astrocytes or oligodendrocytes resulted in neuronal dysfunction, indicating key roles for miRNAs (Tao et al., 2011; Shin et al., 2009). Here, we present evidence that activity of miRNA *mir-263a* is required for healthy nervous system

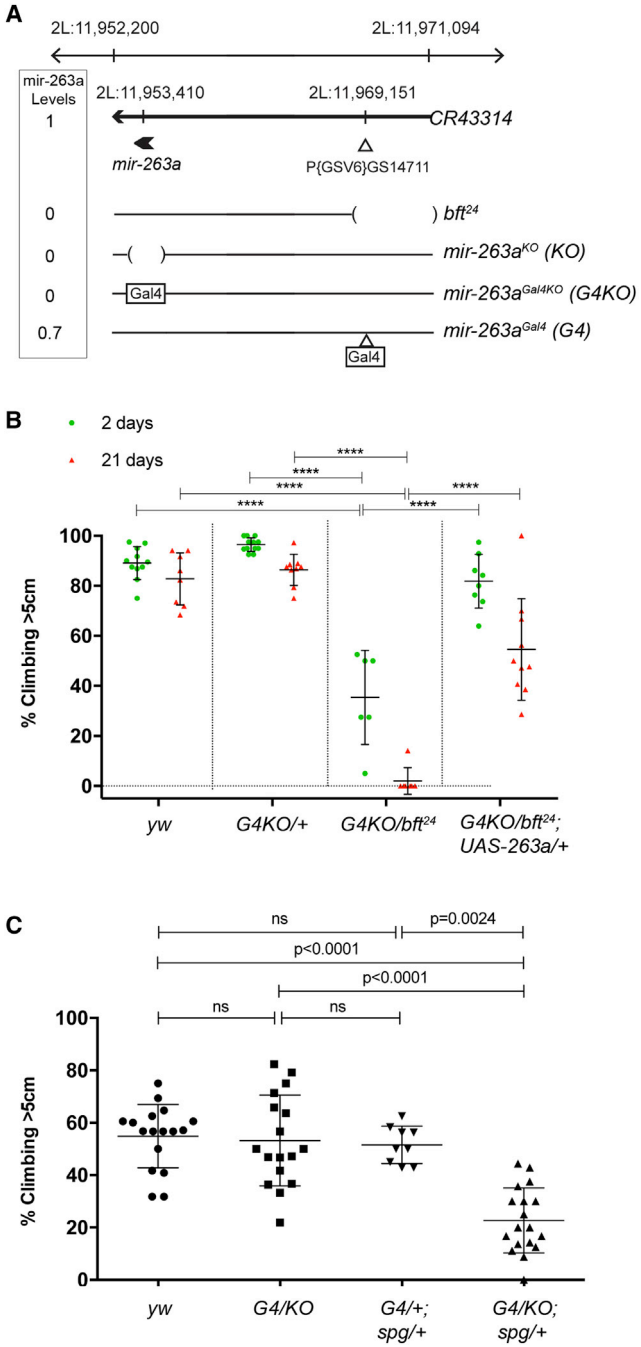


Figure 1. Movement Defect Phenotype in *mir-263a* Mutants

(A) Diagram of the *mir-263a* locus showing the *CR43312* non-coding RNA and the insertion site of the GS14711 P element. Deletions are shown as gaps flanked by parentheses. Parentheses after allele designations show the short names used in other figures. Box at left: relative level of *mir-263a* expression measured by miRNA qPCR (Figure S1).

(B) Climbing assay at 2 and 21 days. Data were analyzed using a two-way ANOVA with Tukey's multiple comparisons test. ****p < 0.0001. p < 0.0001 when comparing mutants at 2 and 21 d. *G4KO*, *mir-263a*^{Gal4KO}; *UAS-263a*, UAS-*mir-263a*.

(C) Climbing assay at 35 days. Expression of the sponge was effective when one copy of the *mir-263a* gene was removed to lower the starting level of miRNA. p values were determined using the Kruskal-Wallis test with Dunn's multiple comparisons post hoc test. Sponge, UAS-*mir-263a* sponge transgene.

See also Figure S1.

function by limiting glutamate receptor levels in a subset of glia. Excess glutamatergic signaling in *mir-263a* mutants leads to excitotoxic glial death and movement defects. Active miRNA-mediated modulation of glutamate sensitivity is therefore important in glia as well as neurons and might be linked to glio-degenerative disease and movement disorders.

RESULTS

Early-Onset, Age-Progressive Climbing Defects in *mir-263a* Mutants

In order to identify miRNAs with functional roles in the nervous system, we carried out a climbing assay screen on a collection of *Drosophila* miRNA mutants (Chen et al., 2014) at 2, 14, and 21 days of age. *mir-263a* was selected for further study because it exhibited a climbing defect that worsens progressively with age. Figure 1A and Table S1 describe the *mir-263a* mutant alleles and Gal4 drivers used in this study. The sequences encoding *mir-263a* are located near the 3′ end of the long non-coding transcript, CR43314. *bft*^{24} is a deletion encompassing the start of this transcription unit that reduces *mir-263a* expression (Hilgers et al., 2010). A deletion allele removing the miRNA sequences (*mir-263a*^{KO}) and a Gal4 knockin allele in which the miRNA was replaced by Gal4 (*mir-263a*^{Gal4KO} or *mir-263aKO-Gal4*) were previously generated by targeted homologous recombination (Hilgers et al., 2010). Fewer than 50% of *bft*^{24}/*mir-263a*^{Gal4KO} mutant males were able to climb normally at 2 days of age (Figure 1B). This defect became markedly worse with age, with almost none able to climb at day 21 (Figure 1B). These mutant flies stayed at the bottom of the vial, shaking and trembling (see Movie S1). Climbing ability was significantly improved by restoring expression of the miRNA in the *mir-263a*^{Gal4KO}/*bft*^{24} mutant background (Figure 1B). Similar results were obtained with female flies and with two other combinations of mutant alleles: *mir-263a*^{Gal4KO}/*mir-263a*^{KO} and *mir-263a*^{KO} / Df(2L)BSC323 (Figure S1). We carried out subsequent experiments with the *bft*^{24}/*mir-263a*^{Gal4KO} mutant combination. Climbing assays for experiments done in this *bft*^{24}/*mir-263a*^{Gal4KO} mutant background were carried out at 18 days. Testing at this age produced consistent phenotypes, which could be made worse by reducing miRNA activity or improved by increasing miRNA activity.

qPCR showed that *mir-263aKO-Gal4*-driven expression of UAS-*mir-263a* produced only ~20% of the normal level of the miRNA (Figure S1). This level of expression was sufficient to only partially rescue the climbing phenotype. In order to generate a stronger Gal4 line, we used P element replacement to introduce a Gal4 P element at a position near the 5′ end of CR43314 (Figure 1A). The resulting *mir-263a*^{Gal4} (or *mir-263a-Gal4*) allele expressed *mir-263a* at ~70% of the level of control flies, indicating that it is a mild hypomorph (Figures 1A and S1). The *mir-263a*^{Gal4} insertion in *trans* to the knockout produced

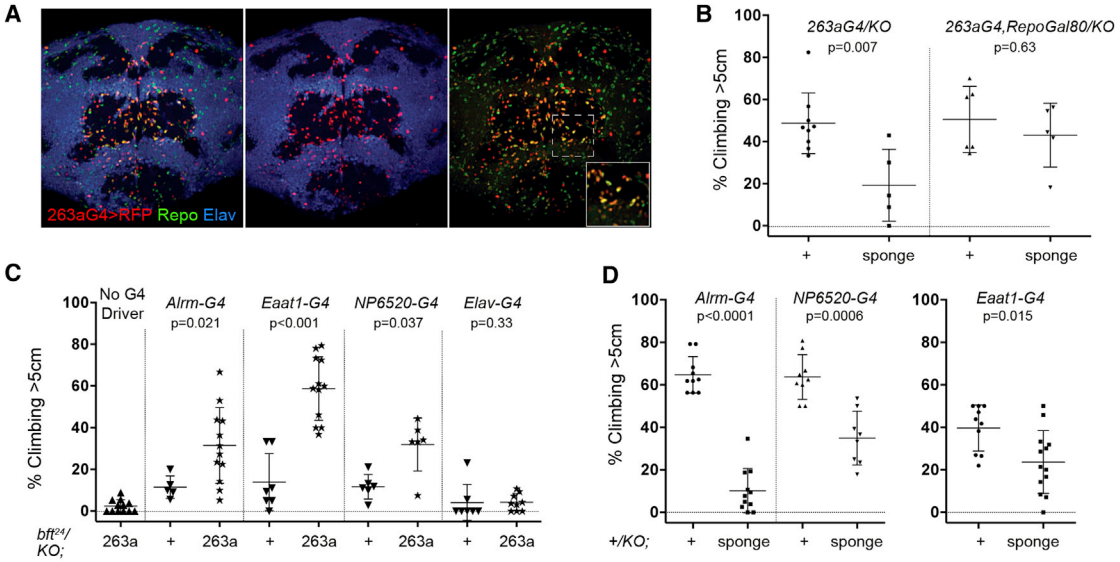


Figure 2. *mir-263a* Is Required in Glia

(A) Maximum projection (anterior view) of a series of optical sections of an adult brain expressing UAS-histone-RFP under *mir-263a-Gal4* control. Glia were labeled with anti-Repo. Neurons were labeled with anti-Elav. Overlap between *mir-263a-Gal4* and Elav was limited to a few cells (see Figure S2). The inset shows a close-up of the overlap in expression between *mir-263a-Gal4* and Repo.

(B) Climbing assay at 35 days. Left: *263aG4/KO* represents the hypomorphic *mir-263a*^{Gal4} in *trans* to the *mir-263a*^{KO} allele. *mir-263a-Gal4* was used to express the miRNA sponge in *mir-263a*-expressing cells. Right: *RepoGal80* was used to block Gal4 activity in glia. p values were determined using the Mann-Whitney test.

(C) Climbing assay at 18 days. Flies were mutant (*bft*^{24}/*mir-263a*^{KO}) and carried the Gal4 (G4) drivers indicated. "263a" indicates the UAS-*mir-263a* transgene introduced into the mutant background using the indicated Gal4 driver; + indicates control chromosome. p values were determined using the Mann-Whitney test.

(D) Climbing assay at 35 days. Flies carried *mir-263a*^{KO} (*KO*) allele in addition to the Gal4 (G4) drivers. + indicates a control chromosome. p values were determined using the Mann-Whitney test.

263aG4, *mir-263a*^{Gal4}; *Sponge*, *mir-263a-sponge*. See also Figure S2.

no discernable climbing defect compared with controls. Climbing defects were observed when *mir-263a* levels were further lowered by *mir-263a-Gal4*-directed expression of a *mir-263a* miRNA sponge (Figure 1C).

mir-263a Is Required in Astrocyte-like and Ensheathing Glia

To visualize the domain of *mir-263a* expression, we crossed the *mir-263a*^{Gal4} insertion allele with a UAS-histone-RFP transgene. *mir-263a-Gal4* activity showed considerable overlap with Repo, a glial transcription factor (Figure 2A). Co-expression with the neuronal marker Elav accounted for fewer than 2% of cells expressing *mir-263a* (Figure S2). This expression pattern was similar to that seen with *mir-263aKO-Gal4* (Figure S1).

To ask if *mir-263a* acts in glia, we used Repo-Gal4 to express the *mir-263a* sponge transgene selectively in glia. When expressing the sponge under *mir-263a-Gal4* control, we found that it was necessary to lower the starting level of the *mir-263a* miRNA, by introducing one copy of the *mir-263a*^{KO} allele, in order to reveal a phenotype (Figure 1C). However, we could not use this approach with Repo-Gal4, because introducing one copy of the *mir-263a*^{KO} allele into the Repo-Gal4 background on its own caused a strong climbing defect, suggesting a genetic interaction in the absence of the sponge transgene (Figure S2). As an alternative, we used Repo-Gal80 to selectively block Gal4 activity in the glial subset of the *mir-263a-Gal4* expression domain, while allowing activity in other cells. When Gal4 activity was blocked in the glial subset of the *mir-263a*-expressing cell population, the climbing defect caused by expression of the miRNA sponge was suppressed (Figure 2B). This provided evidence that *mir-263a* activity is required in glia.

We used two additional genetic strategies to explore the requirement for *mir-263a* in different types of glia and neurons. First, we used Gal4 drivers to restore expression of *mir-263a* in selected glial and neuronal cells in the *mir-263a* mutant background. Without any Gal4 driver, UAS-*mir-263a* did not rescue the climbing defect (Figure 2C). Expression of the miRNA with the glia drivers Eaat1-Gal4 or Alrm-Gal4 (Doherty et al., 2009) was sufficient to restore climbing (Figure 2C). Alrm-Gal4 is an astrocyte driver, and *Eaat1* is expressed predominantly in astrocytes, with some expression in ensheathing and cortex glia (Doherty et al., 2009) (Figure S3). Expression of the miRNA with the ensheathing glia driver NP6520-Gal4 (Awasaki et al., 2008) also improved climbing, but expression with the neuronal driver Elav-Gal4 did not (Figure 2C). We also expressed the *mir-263a* sponge in each glial subtype in the *mir-263a*^{KO}/+ heterozygous background to ask if the miRNA was necessary in each glial subtype. Depleting the miRNA from astrocyte-like glia using Eaat1-Gal4 and Alrm-Gal4 or from ensheathing glia with NP6520-Gal4 produced climbing defects (Figure 2D). Depletion of the miRNA with the cortex glia driver NP2222-Gal4 was ineffective (Figure S2). These results indicate that *mir-263a* is

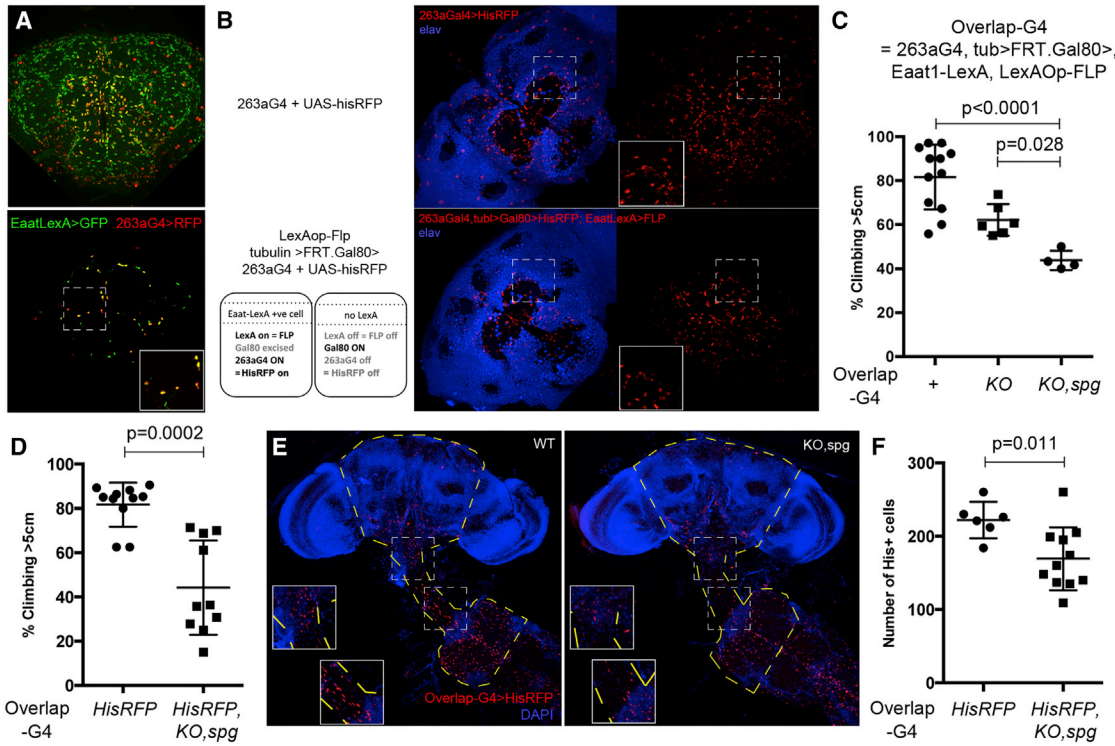


Figure 3. *mir-263a* Is Required in a Subset of Astrocyte-like and Ensheathing Glia Expressing Both *Eaat1* and *mir-263a*

(A) Adult brains expressing *mir-263a-Gal4*–driven UAS-histone-RFP (red) and Eaat1-LexA-driven LexAOp-nls.GFP (green). Top: maximum projection (anterior view). Bottom: overlap between the two drivers in a single section. The inset shows a close-up of the dashed region.

(B) Left: scheme for generation of a driver expressing Gal4 activity only in cells expressing both *mir-263a-Gal4* and Eaat1-LexA (see text for details). Right top: complete *mir-263a-Gal4* expression domain. Right bottom: overlap between cells expressing Eaat1-LexA and *mir-263a-Gal4*. The insets show close-ups of the dashed regions.

(C) Climbing assay at 35 days. All flies expressed the driver for the overlap population, Overlap-Gal4 (B): *mir-263a*^{Gal4}, *αTub84B > FRT.Gal80 >* ; *Eaat1-LexA*, *LexAOp-FLP*, with the addition of the indicated transgenes. + indicates a wild-type copy of *mir-263a*; *KO* indicates *mir-263a*^{KO}; and *KO*, *spg* indicates *mir-263a*^{KO} with *mir-263a* sponge. p values were determined using an ordinary one-way ANOVA ($p < 0.05$) followed by a protected Fisher's least significant difference (LSD) test.

(D) Climbing assay at 35 days. All flies expressed the driver for the overlap population, Overlap-Gal4 (B), with addition of the indicated transgenes. *KO*, *spg* indicates *mir-263a*^{KO} with *mir-263a* sponge. p values were determined using the Mann-Whitney test.

(E) Representative images of dissected CNS from 35 day control and *mir-263a*-depleted flies from (D), showing the areas for quantification in (F). Images are maximum projections (posterior view for optimal imaging of the connective). Genotypes: WT, *Overlap-Gal4*, *UAS-hisRFP*; and KO, spg, *Overlap-Gal4/ mir-263a*^{KO}; *UAS-hisRFP*, UAS-*mir-263a-sponge.* The insets show close-ups of the respective dashed regions.

(F) Quantification of the Eaat1-LexA/*mir-263a-Gal4* overlap glial population of animals from (D). Each data point represents one fly. p values were determined using the Mann-Whitney test. Control sample average: 222 ± 10 (SEM; n = 6) His+ cells. *mir-263a*-depleted sample average: 169 ± 13 (n = 11).

See also Figure S3.

required in astrocyte-like and ensheathing glia to support climbing behavior.

mir-263a Is Required in a Population of Glia Expressing Both *mir-263a* and *Eaat1*

Given that the *mir-263a-Gal4* driver is expressed in a subset of glia and that expression of the miRNA in Eaat1-Gal4 expressing cells rescued the phenotype, we infer that the miRNA acts in the population of glia in which these two Gal4 drivers overlap. To compare their expression, we made use of an Eaat1-LexA driver to drive nuclear-GFP expression together with *mir-263a-Gal4*-directed expression of histone-RFP. As a control for Eaat1-LexA, we first compared its expression with that of Eaat1-Gal4. All Eaat1-LexA cells expressed Eaat1-Gal4 (Figure S3). The Eaat1-LexA and *mir-263a-Gal4* drivers were each expressed in hundreds of neuropil glia, but the population in which the two overlap was considerably smaller (Figure 3A).

If *mir-263a* activity is required in this overlap population, selectively depleting the miRNA from these cells with the sponge transgene should phenocopy the climbing defect in the mutant. In order to do this experiment, we required a means to allow Gal4 activity selectively in the overlap population. This was done by ubiquitously expressing Gal80 from a transgene in which the Gal80 sequence was flanked by FLP recombinase target sites. These sites were oriented so that FLP recombinase would excise the Gal80 cassette, allowing Gal4 activity. We used Eaat1-LexA to drive expression of FLP recombinase together with *mir-263a-Gal4*-directed expression of RFP (see Figure 3B). This strategy

proved to be effective in limiting the extent of *mir-263a-Gal4* expression (Figure 3B). Glia expressing both *mir-263a-Gal4* and Eaat1-LexA were located in the central brain and cervical connective and ventral nerve cord (VNC) (Figures 3B and 3E). Expressing the *mir-263a* sponge selectively in these cells produced a climbing defect (Figure 3C). Although the climbing defect was mild compared with the null mutant, the difference between control and experimental samples was significant, indicating that *mir-263a* acts in the population of glia in which its expression overlaps with that of *Eaat1*. We note that this does not exclude the possibility that *mir-263a* is also required in other cells.

Although Eaat1-LexA co-expresses strongly with Alrm-Gal4, it also shows overlap with NP6520-Gal4 (Figure S3). Alrm-Gal4 is expressed predominantly in astrocytes and NP6520 in ensheathing glia, and both drivers are expressed in the functional domain of *mir-263a* (Figures 2C and 2D). Eaat1-Gal4 and NP6520Gal4 are also expressed in cortex glia; however, our data suggest that *mir-263a* function in this population does not contribute to the climbing defects (Figure S2). Hence, *mir-263a* functions in a subset of astrocytes and ensheathing glia.

To examine the consequences of selective depletion of *mir-263a* on the population of glia expressing both *mir-263a* and *Eaat1*, we used the FLP-out Gal80 strategy (Figure 3C), adding UAS-histone-RFP as a reporter. The flies expressing both sponge and histone-RFP in the heterozygous background exhibited climbing defects compared with flies expressing histone-RFP alone (Figure 3D). Dissection of the brain and cervical connective and VNC revealed ~20% decrease in the number of RFP-positive nuclei when *mir-263a* was depleted (Figures 3E and 3F). Therefore, depletion of *mir-263a* from this specific population causes a loss of these glia and a concomitant climbing defect.

mir-263a Limits Glutamatergic Signaling

miRNAs typically downregulate their targets. To identify *mir-263a* targets, we carried out expression profiling on RNA isolated from heads of control flies, $mir\text{-}263a^{Gal4KO}/mir\text{-}263a^{KO}$ mutants, and the rescued $mir\text{-}263a^{Gal4KO}/mir\text{-}263a^{KO}$ mutants expressing the UAS-*mir-263a* transgene. We selected for further analysis 71 transcripts that were upregulated in the mutant and reduced again in the rescued mutant and that contained plausible *mir-263a* target sites (Table S2). Another 20 computationally predicted targets with nervous system functions were also tested.

If upregulation of a target transcript contributes to the mutant phenotype, reducing its expression should suppress that phenotype. We made use of UAS-RNAi transgenes as well as deficiencies or P-element mutant alleles to individually reduce activity of the 91 candidate genes in the $mir\text{-}263a^{Gal4KO}/bft^{24}$ mutant background. Only one target showed significant suppression of the mutant phenotype using both genetic tests. Depletion of *CG5621* by RNAi improved climbing in the mutant background (Figure 4A; Movie S1). Similarly, introducing one copy of a *CG5621* P element insertion allele improved climbing (Figure 4A). *CG5621* (previously annotated on Flybase as *DKaiRIC*) encodes a glutamate receptor that is most similar to members of the *GRIK* human kainate receptor family. We henceforth refer to it as *Grik*.

Like other glutamate receptor types, kainate receptors are tetramers. Methionine 589 in human GluK1 has been identified as a key residue in the conserved pore domain required for ion binding, and mutation of M589 to arginine produces a dominant-negative subunit that blocks endogenous receptor function when incorporated into a heteromeric complex (Robert et al., 2002). On the basis of sequence alignment, we infer that leucine 601 in the *Drosophila* Grik protein corresponds to mammalian M589 (Figure 4B). We prepared a similar $Grik^{L601R}$ dominant-negative transgene and introduced this into the $mir\text{-}263a^{Gal4KO}/bft^{24}$ mutant background. Expression of $Grik^{L601R}$ improved climbing performance of the mutant (Figure 4B). Together with the preceding experiments in which lowering *Grik* expression suppressed the climbing defect, we conclude that limiting *Grik* activity can improve climbing performance in *mir-263a* mutants. *Grik* expression was monitored using a Gal4 P-element insertion allele in CG5621 (*Grik*-Gal4). The Gal4 line was largely expressed in glia (Figure S4).

Although *Grik/CG5621* was the only glutamate receptor detected among the transcripts that were significantly upregulated in the expression profiling of *mir-263a* mutant brains, potential *mir-263a* target sites were predicted in the 3′ UTRs of seven other glutamate receptors (Figure S5). We tested depleting expression of these receptors using RNAi in the *mir-263a* mutant background. Depletion of *mGluR*, *clumsy*, and *GluRIIE* was ineffective, but RNAi-mediated knockdown of the NMDA receptors *Nmdar1* and *Nmdar2* and the kainate receptors *CG11155* and *Ekar* suppressed the climbing defect (Figure 4C). Expression of the RNAi transgenes for *Grik*, *Nmdar1*, and *Nmdar2* with *mir-263a-Gal4* in an otherwise wild-type background did not cause climbing defects (Figure S5).

Expression levels of *mir-263a* targets are expected to be upregulated in *mir-263a* mutants. The levels of *Nmdar1*, *Nmdar2*, *CG11155*, and to a smaller extent, *Grik*, increased in RNA isolated from *mir-263a* mutant heads when assayed by qPCR, and this was offset in the rescued mutant (Figure 4D). However, the fold change observed was low. This would be expected if the affected cells constituted a limited subset of the expression domain of these receptors. To address this, we measured transcripts in RNA recovered selectively from *Eaat1*-positive glia by thio-labeling RNA using Eaat1-Gal4-driven uracil phosphoribosyltransferase (Miller et al., 2009). In RNA from *Eaat1*-expressing glia, the levels of *Nmdar1*, *Nmdar2*, and *Grik* increased by 2.4-fold, 3.4-fold, and 1.5-fold, respectively, in *mir-263a* mutants compared with controls (Figure 4E). Upregulation of *Nmdar1* transcript was accompanied by an increase in Nmdar1 protein in the mutant. Nmdar1 protein normally takes on a speckled, ubiquitous distribution, representing weak expression in most neurons (Wu et al., 2007; Xia et al., 2005). Depletion of *mir-263a* caused upregulation of Nmdar1 protein in a subset of *mir-263a-Gal4*-expressing glia in the nervous system, including in the central brain (Figure 4F). This was not observed in control samples (Figure 4F).

To determine if the climbing defects seen in *mir-263a* mutants could be caused by glial cell loss, we asked if the number of *mir-263a-Gal4*, Eaat1-LexA overlap cells (Figure 3B) was rescued by lowering glutamate receptor levels. When the *mir-263a* sponge was expressed in this overlap population, climbing defects were again observed, and glial cell number decreased (Figure 4G). When *Grik* was downregulated by RNAi in this overlap

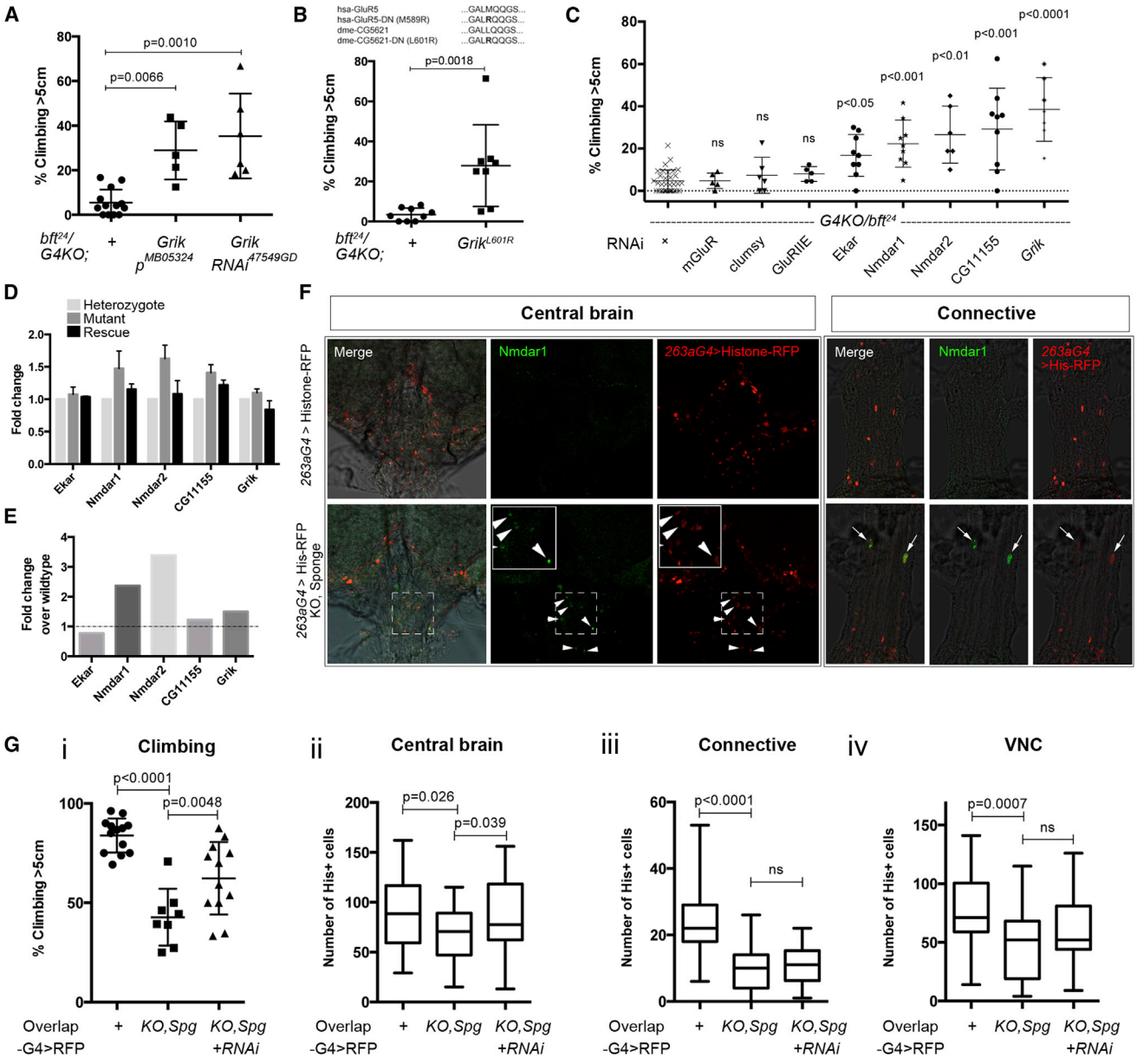


Figure 4. Functional Rescue by Depletion of Glutamate Receptors

(A–C) Climbing assay at 18 days. All flies were in the mutant background (*mir-263a*^{Gal4KO}/*bft*^{24}). + indicates mutant alone. (A) *Grik* p^{MB05324} (*Mi{ET1} CG5621*^{MB05324}) has a P element inserted into *Grik* (exon 7). RNAi^{47549GD} is a UAS-RNAi transgene targeting *Grik*. p values were determined using the Kruskal-Wallis test. (B) *Grik*^{L601R} indicates a UAS transgene for a dominant-negative *Grik*. p values were determined using the Mann-Whitney test. (C) RNAi: the target of each RNAi transgene is indicated. p values were determined using the Kruskal-Wallis test with Dunn's multiple comparisons post hoc test.

(D) Glutamate receptor levels in *mir-263a* mutant brains. Transcript levels of glutamate receptors that were putative targets for *mir-263a* (from C) measured by qPCR using RNA from *mir-263a*^{Gal4KO}/+ heterozygote, *mir-263a*^{Gal4KO}/*bft*^{24} mutant, and *mir-263a*^{Gal4KO}/*bft*^{24}; *UAS-263a*/+ rescued mutant heads. Data are from three biological replicates.

(E) Glutamate receptor levels in Eaat1-Gal4-expressing cells in *mir-263a* mutant brains measured by qPCR using RNA from Eaat1-Gal4-expressing cells in control (*Eaat1-Gal4*/+, *UAS-UPRT*/+) and mutant (*Eaat1-Gal4*, *bft*^{24}/*mir-263a*^{KO}; *UAS-UPRT*/+) heads.

(F) Single optical sections of anti-Nmdar1 (green) in the CNS (posterior views) of *mir-263a*^{Gal4}/+; *UAS-Histone-RFP*/+ control cells compared with *mir-263a*^{Gal4} cells using the *mir-263a*^{KO} allele with the *mir-263a* sponge to further lower *mir-263a* levels (KO, spg, *mir-263a*^{Gal4}/*mir-263a*^{KO}; UAS-histone-RFP/UAS-*mir-263a*-sponge). The insets show close-ups of the respective dashed regions.

(G) Cell-type-specific depletion of *Grik* rescues climbing defect and cell numbers of *mir-263a-Gal4*/Eaat1-LexA overlap cells (Overlap-G4) depleted of *miR-263a*. Climbing assays (i) and cell quantification (ii–iv) in central brain (ii), cervical connective (iii), and ventral nerve cord (VNC) (iv) performed at 35 days. In (i), each data point represents a biological replicate of 15–20 flies. In (ii–iv), each data point represents the dissected CNS region from a single fly (n = 25–30 brains for each of the nine data sets). All flies expressed Overlap-Gal4 driving UAS-Histone-RFP (*mir-263a*^{Gal4}, *αTub84B > FRT.Gal80 > /Eaat1-LexA*; *LexAOp-FLP/UAS-hisRFP*),

(legend continued on next page)

glial population, the climbing defects were suppressed (Figure 4Gi). Interestingly, central brain glia were rescued under these conditions (Figure 4Gii), although cells were lost in all regions in the mutant. Cell numbers were partially restored in the cervical connective and VNC, but this was not statistically significant (Figures 4Giii and 4Giv). These experiments support the hypothesis that the climbing defects seen in *mir-263a* mutants are due to the loss of glia, primarily in the central brain, caused by excess glutamate receptor levels.

Modulating Glial Cell Membrane Polarization

Can modulating glutamate signaling by other means affect the severity of the mutant phenotype? Excitatory amino acid transporters remove extracellular glutamate from the synaptic cleft to prevent glutamate accumulation that could lead to excitotoxic cell death. We expressed UAS-*Eaat1* in its endogenous domain under Eaat1-Gal4 control to lower extracellular glutamate levels. This improved climbing performance in the *mir-263a* mutant (Figure 5A).

Ligand binding to glutamate receptors typically leads to cell depolarization. We asked if reducing depolarization of *mir-263a*-expressing cells by overexpression of the potassium channel Kir2.1 (KCNJ2; Baines et al., 2001) could rescue the climbing defect in *mir-263a* mutants. This proved to be effective (Figure 5B). Reciprocally, to mimic the effects of excessive glutamate signaling, we used a temperature-sensitive UAS-*TrpA1* channel (Pulver et al., 2009) to selectively depolarize *mir-263a*-expressing cells. This caused a severe climbing defect, similar to what was observed in the *mir-263a* mutant (Figure 5C). We then examined the number of *mir-263a*-expressing cells when TrpA1 was activated and found that these flies had a ~20% fewer *mir-263a*-expressing cells in the central brain, but not in the connective or VNC (Figures 5D and 5E). Thus, mimicking excess glutamate receptor activation by excess depolarization appears to compromise the survival of these central brain cells.

These experiments support the hypothesis that *mir-263a*-expressing glia show increased sensitivity to ambient levels of extracellular glutamate. This can be offset by increased glutamate scavenging, reduced glutamate receptor expression or activity, and modulating membrane depolarization in a cell-type-specific manner. We hypothesized that the loss of cells accompanying the increased glutamate sensitivity was a result of glutamate-induced excitotoxic cell death. Expression of UAS-*Diap1* to block cell death in the mutant improved climbing (Figure 5F). Our data support a mechanism of glutamate-induced excitotoxic cell death in *mir-263a* mutant glia, caused by misregulation of glutamate receptors.

DISCUSSION

Glutamate is an important neurotransmitter at many excitatory synapses. Astrocytic glia protect synapses and help to maintain neuronal function by clearing glutamate. This allows rapid cycles of synaptic neurotransmission and protects neurons from the effects of extracellular glutamate buildup (Anderson and Swanson, 2000; Rothstein et al., 1996). A growing body of evidence points to glutamate signaling in glia themselves (Cornell-Bell et al., 1990; Lalo et al., 2006). Although the functional impact of this signaling is not well understood for the most part (reviewed by Kolodziejczyk et al., 2010), there are some indications of specific functions. For example, glutamate has been reported to regulate glial glutamate transporter expression, suggesting active feedback to control extracellular glutamate levels (López-Bayghen et al., 2003). One study found that loss of AMPA receptors resulted in retraction of glial processes from synapses, leading to impairment in fine motor coordination (Saab et al., 2012). Evidence has also been presented for glutamatergic regulation of signaling from glia to neurons (Mothet et al., 2005).

Our findings provide evidence that miRNA-mediated regulation of glutamate receptor levels in astrocyte-like glia and ensheathing glia in *Drosophila* is required to prevent excitotoxic death of these glia. The movement defects in *mir-263a* mutants were rescued by preventing depolarization of these glia, suggesting that excess glutamate signaling was the cause of glial death. Both the cell loss and concomitant climbing defects in the mutant were rescued by lowering glutamate receptor levels in these cells. Increasing glutamate scavenging and blocking cell death also rescued the climbing defect in the mutant. Together, these findings provide evidence that elevated glutamate sensitivity was causative. In addition, the rescue of cells in the overlap population due to reduced target expression correlated with restored climbing ability. *mir-263a* appears to modulate glutamate responsiveness in these cells by regulating expression of several glutamate receptors. The targeting of groups of glutamate receptors by one miRNA might be an efficient mode of regulation, because subunits may be interdependent for the functional assembly of heteromeric receptors (Qin et al., 2005). The endogenous functions of these glutamate receptors in glia will be an interesting topic for future study.

miRNA-mediated repression of glutamate receptor subunits has been shown to be neuroprotective (Harraz et al., 2012). Alterations in glutamate receptor levels may change receptor composition, altering channel properties (Sans et al., 2003) and glutamate response (Harraz et al., 2012). For example, AMPA channels lacking GluR2 show increased susceptibility to excitotoxicity (Hollmann et al., 1991; Peng et al., 2006). Notably, motifs that control voltage-dependent Mg^{2+} block are only somewhat conserved in *Drosophila* NMDARs, suggesting that they may exhibit higher Ca^{2+} permeability (Xia et al., 2005), which is associated with excitotoxic cell death (Arundine and Tymianski, 2003; Randall and Thayer, 1992). These subtle receptor abnormalities can lead to chronic, slow excitotoxicity (Warmus et al., 2014; Beal, 1992). It is important to distinguish the cell-autonomous

with the addition of the indicated transgenes: *KO*, *mir-263a*^{KO}; *Spg* = *mir-263a* sponge; RNAi, UAS-RNAi line against *Grik*. p values were determined using one-way ANOVA ($p < 0.0001$) followed by a protected Fisher's least significant difference test. In the central brain, control samples had an average of 87 ± 6.7 (SEM, n = 28) His+ cells; *miR-263a*-depleted average: 68 ± 4.5 (n = 30); *Grik*-RNAi rescue: 85 ± 6.7 (n = 30).
See also Figure S4.

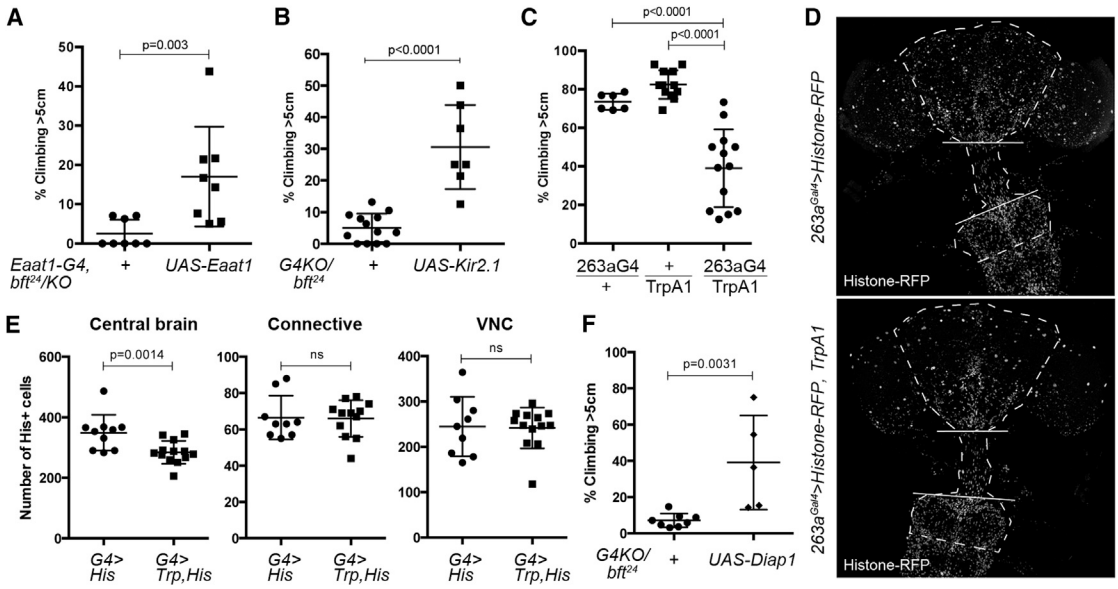


Figure 5. Phenotypic Modulation by Manipulating Extracellular Glutamate Levels, Membrane Polarization of Glia, and Blocking Cell Death

(A) Climbing assay at 18 days. All flies carried *bft*^{24} recombined with Eaat1-Gal4 in *trans* to *mir-263a*^{KO}. + indicates mutant. p values were determined by Mann-Whitney test.

(B) Climbing assay at 18 days. All flies carried *mir-263a*^{Gal4KO} in *trans* to *bft*^{24}. p values were determined by Mann-Whitney test.

(C) Climbing assay at 11 days. Flies were reared at 18°C, shifted to 29°C 3 days before eclosion, and kept at 29°C until tested. 263aG4, *mir-263a-Gal4*. TrpA1 indicates a UAS-*TrpA1* transgene. p values were determined using an ordinary one-way ANOVA ($p < 0.0001$) followed by a protected Fisher's least significant difference test.

(D) Representative images of dissected whole CNS from control flies and flies expressing activated TrpA1 in *mir-263a-Gal4*-expressing cells from (C), demonstrating the demarcation of areas for quantification in (E).

(E) Quantification of *mir-263a-Gal4*-expressing glia in animals from (C). p values were determined using the Mann-Whitney test. In the central brain, control samples had an average of 349 ± 19 (SEM, n = 10) His+ cells; TrpA1-activated samples had 284.5 ± 10.4 (n = 13). There was no significant difference in the cervical connective and ventral nerve cord.

(F) Climbing assay at 18 days. All flies carried *mir-263a*^{Gal4KO} in *trans* to *bft*^{24}. p values were determined by Mann-Whitney test.

effects due to increased glutamate sensitivity, like those observed in this study, from the widespread excitotoxicity to neurons and glia that occurs due to excessive glutamate release or impaired glutamate clearance, which can affect neighboring cells, as was seen in the case of *miR-1000/137* (Verma et al., 2015) and in stroke and traumatic brain injury (Beal, 1992). It would be interesting to find out if slow glial excitotoxicity due to cell-autonomous glutamate receptor upregulation may be a relevant contributing mechanism to human movement disorders.

Neurons and muscles use miRNAs to regulate glutamate receptor levels (Harraz et al., 2012; Letellier et al., 2014; Saba et al., 2012; Karr et al., 2009). Neurons, with their elongated and highly branched morphology, traffic mRNAs over long distances into axons and dendrites, accumulating compartmentalized pools of mRNAs for onsite translation in response to local stimuli (Cajigas et al., 2012). There is accumulating evidence that miRNAs locally regulate mRNAs post-synaptically, to modulate synaptic plasticity (Schratt et al., 2006) as well as pre-synaptically to modulate glutamate release (Verma et al., 2015). Astrocytes, like neurons, have irregular and complex morphologies and may also exhibit subcellular trafficking and localized translation of transcripts (Gerstner et al., 2012). As in axons, miRNAs are well suited to confer local regulation of target mRNAs at distal sites in glia with elongated morphologies.

How might loss of this specific glial population lead to the observed climbing defects? It had previously been shown that acute elimination of neuropil glia causes misguidance and abnormal fasciculation of secondary axon tracts (Spindler et al., 2009). A separate study showed that elimination of astrocytes resulted in changes in synapse number (Muthukumar et al., 2014). Adults that emerged under these conditions exhibited severe motor defects and extremely short lifespan (Muthukumar et al., 2014; Spindler et al., 2009). In this study, we show that loss of *mir-263a* led to loss of a small, specific population of astrocyte and ensheathing glia and that loss of this population is associated with severe climbing defects. It would be reasonable to assume that loss of these glia impacts the function of interacting neurons. Variation in the severity of this indirect effect likely explains why some mutant animals climb better than others.

mir-263a is similar in sequence to the mammalian *mir-183/-96/-182* cluster. Interestingly, *mir-182* is expressed in astrocytes and upregulated in glioblastoma (Kouri et al., 2015). *mir-182* is also predicted to target the kainate receptor *GRIK3*, AMPA receptors *GRIA1* and *GRIA3*, the metabotropic glutamate receptor *GRM5*, and glutamate receptor *GRID1* (http://www.targetscan.org). *mir-182* is upregulated in glial Schwann cells in response to nerve injury (Yu et al., 2012). It will be of interest

to learn whether *mir-183/-96/-182* have a comparable glioprotective function through regulating glutamate receptors in mammalian glia. The potent effects on glutamate sensitivity observed from loss of a single miRNA, as seen in this study, suggests potential use of miRNAs to regulate glutamate receptor expression for treatment of disease.

EXPERIMENTAL PROCEDURES

Fly Strains

Flies and crosses were reared at 25°C, 70% humidity in an environmentally controlled incubator on a 12 hr light-dark schedule unless otherwise indicated. Control flies were *yw* unless otherwise indicated. Data are from males unless otherwise indicated.

mir-263a^{KO}, *mir-263a*^{Gal4KO}, and *UAS-mir-263a* are described in Hilgers et al. (2010). *Df(2L)BSC323* (removes *mir-263a*), *Mi{ET1}CG5621[MB05324]* (*CG5621* allele), *P{w[+m*] = GAL4}repo* (repo-Gal4), *P{GAL4-elav.L}2* (elav-Gal4), *P{Eaat1-GAL4.R}2* (Eaat1-Gal4), *P{8XLexAop2-FlpL}attP2* (LexAOp-FLP), *P{w[+m*] = lexA-2xhrGFP.nls}2a* (LexAOp-nlsGFP), *P{alphaTub84B(FRT.GAL80)}2* (tub > FlpoutGal80 >), *P{GMR75H11-lexA}attP40* (Eaat1-LexA), *P{y[+t7.7] w[+mC] = UAS-TrpA1(B).K}attP2* (UAS-TrpA1), *P{w[+mC] = UAS-Hsap\KCNJ2.EGFP}7* (UAS-Kir2.1), and *P{UAS-Eaat1.Exel}3* (UAS-Eaat1) were from Bloomington *Drosophila* Stock Center (Indiana University). *P{GawB}NP6520* (NP6520-Gal4), P*{GawB}Akap200*^{NP2222} (NP2222), *P{GawB}NP577* (NP577-Gal4), and *P{GSV6}GS14711* (*mir-263a* insertion used for P-element replacement) were from DGRC. Alrm-Gal4 was provided by Marc Freeman. *bft*^{24} was provided by Rolf Bodmer. RepoGal80 was provided by Tzumin Lee. ItGal4$^{3.1}$ was provided by David Foronda. *P(KK* 102270*)v103736*(*mGluR*-RNAi), *P(KK104645)v105870* (*clumsy*-RNAi), *P(GD16345)v49547* (*GluRIIE*-RNAi), *P(KK*111536*)v102351* (*Ekar*-RNAi), *P(KK* 107519*)v104773* (*Nmdar1*-RNAi), *P(KK*105030*)v100883* (*CG11155*-RNAi), *P(GD* 3192*)v12189*(*Nmdar2*-RNAi), and *P(GD*3092*)v47549* (*CG5621/Grik*-RNAi), were from the Vienna *Drosophila* Research Center.

Climbing Assays

Crosses were set up with 25 females per bottle and flipped every 2 days to prevent overcrowding. Groups of 15–20 males were collected within 24 hr of eclosion and aged without further CO_2 exposure. Flies were flipped onto fresh food every 2–3 days. Each data point represents 15–20 flies in one vial. Climbing experiments were carried out between 3 and 6 pm to minimize circadian differences. Flies were transferred to cylindrical vials measuring 2.5 × 9 cm, with a line at the 5 cm mark, and allowed to acclimate undisturbed for 15–30 min before testing. Flies were lightly tapped down to the bottom of the vial, and the number that crossed the 5 cm mark in 30 s were counted. Vials were placed horizontally and retested 15–30 min later. The average of the two technical replicates for each vial was recorded, and the percentage climbing was plotted as a single point. The knockouts and mutants, being a complete loss of function, show a defect at a younger age than the flies in which miRNA function is partially reduced using the sponge. Thus, climbing analysis of mutants were carried out at 18 days and sponge experiments at 35 days.

P Element Replacement for Generation of *mir-263a*^{Gal4}

The *mir-263a*^{Gal4} allele was made by replacing the P element insertion in *P{GSV6}GS14711* with the Gal4 P-element ItGal4$^{3.1}$ using Δ2-3 recombinase. Candidates were screened for changes to UAS-histone-RFP expression. One candidate with a distinct pattern was found and the insertion mapped by inverse PCR. The insertion was mapped to 2L:11,969,151 indicating that it had replaced the *P{GSV6}GS14711* target.

UAS-*TrpA1*

Crosses were set up with 25 females per bottle and flipped every 3 days. Crosses were kept at 18°C until pupariation (3 days before eclosion) and shifted to 29°C. Flies were collected within 24 hr of eclosion and kept at 29°C. Groups of 15–20 males were kept at 29°C without further exposure to CO_2 and assayed for climbing ability on day 11 post-eclosion.

TU Tagging

Eaat1-Gal4/+; *UAS-UPRT/+* (control) and *Eaat1-Gal4*, *bft*^{24}/*263a*^{KO}; *UAS-UPRT/+* (mutant) flies were raised until 4 days of age. They were placed in vials with 1% agarose for 12 hr and then transferred to food with 1 mM 4-TU (24 hr in the dark). Flies were frozen in liquid nitrogen and agitated to remove the heads. Three hundred heads were used for each sample. Tagged RNA was purified as previously described (Miller et al., 2009) and used for qRT-PCR. Samples were tested to ensure that *repo* mRNA was enriched in the bound fraction and that *elav* mRNA was enriched in the unbound fraction.

Cell Counting

Dissection, fixing, staining, imaging, and analysis were carried out blind. Samples were mounted for view from posterior. Two-micrometer optical sections were taken through the entire depth of each sample. Because UAS-histone-RFP cells were sparse with minimal overlap, a maximum projection was used for counting. Demarcation of central brain, connective, and the first thoracic segment of the VNC was carried out without reference to the histone-RFP channel (see Figure S3) using Nrg (Figure 3) or DIC bright-field (Figures 4 and 5). The histone-RFP channel was then extracted from the selection, and the number of RFP-positive nuclei were counted using the ITCN plugin (Center for Bio-Image Informatics, University of California, Santa Barbara) in ImageJ. The ITCN settings were determined on a control brain in the first experiment so that most visible nuclei were selected with no false positives, and the same setting was applied to all images (width = 9, minimum distance = 4.5, threshold = 2.2). For Figure 3F, only fully intact samples were counted. For Figures 4G and 5E, only intact regions were counted.

Statistical Analysis

Statistical analysis was carried out using Prism 6 (GraphPad Software). Scatter plots were used except if n > 25, in which case box-and-whisker plots were used (the ends of the whiskers represent the minimum and maximum of all the data). When comparing two populations (mutant versus treatment), a two-tailed non-parametric Mann-Whitney test was used. When comparing three or more populations for the purposes of phenotypic characterization (Figures 1B and 1D) or candidate screening (Figures 4A and 4D), a non-parametric Kruskal-Wallis test was used, with Dunn's multiple comparisons post hoc test to determine which were statistically significant outcomes. When comparing three or more populations for testing specific hypotheses (Figures 3C, 4C, 4H, and 5C) one-way ANOVA was used, with a protected Fisher's least significant difference (LSD) test to determine if each comparison was statistically significant (in all cases, the overall ANOVA F ratio was <0.05).

Determination of Sample Sizes

On the basis of our initial sponge results, comparing G4/KO (mean = 56.3, SD = 16.4) with G4/KO, sponge (mean = 20.2, SD = 15.5), the effect size of the miR-263a phenotype was found to be relatively large (Cohen's d = 2.261). The effect size comparing mutant to controls was still larger. To uncover effects at a 0.05 significance level with 80% power, a minimum of five samples in each group was required. Because it was not possible to obtain a technical replicate of each fly's climbing performance in the group, we reported the climbing ability as percentages of a group.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, two tables, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.05.010.

AUTHOR CONTRIBUTIONS

S.S.A., M.X.M.T., and I.K.H.L. conducted the experiments. S.S.A. and S.M.C. designed the experiments, analyzed the data, and wrote the paper.

ACKNOWLEDGMENTS

We thank Kah Junn Tan and Alice Liu for technical support and members of the Cohen lab for insightful discussions. We also thank Rolf Bodmer,

Chia-Lin Wu, Shouzhen Xia, Marc Freeman, Tzumin Lee, and David Foronda for generously sharing reagents. This work was supported by the Institute of Molecular and Cell Biology, Singapore, by A*STAR Joint Council Organisation grant 15302FG149 to S.S.A., and by the Singapore National Research Foundation under its Translational and Clinical Research Flagship Programme (Parkinson's Disease) grant NMRC/TCR/013-NNI/2014 administered by the Singapore Ministry of Health's National Medical Research Council. S.M.C. was supported by NovoNordisk Foundation grant NNF12OC0000552 for part of this work.

Received: December 2, 2016
Revised: March 14, 2017
Accepted: May 2, 2017
Published: May 30, 2017

REFERENCES

Anderson, C.M., and Swanson, R.A. (2000). Astrocyte glutamate transport: review of properties, regulation, and physiological functions. Glia *32*, 1–14.

Arundine, M., and Tymianski, M. (2003). Molecular mechanisms of calcium-dependent neurodegeneration in excitotoxicity. Cell Calcium *34*, 325–337.

Awasaki, T., Lai, S.L., Ito, K., and Lee, T. (2008). Organization and postembryonic development of glial cells in the adult central brain of Drosophila. J. Neurosci. *28*, 13742–13753.

Baines, R.A., Uhler, J.P., Thompson, A., Sweeney, S.T., and Bate, M. (2001). Altered electrical properties in Drosophila neurons developing without synaptic transmission. J. Neurosci. *21*, 1523–1531.

Beal, M.F. (1992). Mechanisms of excitotoxicity in neurologic diseases. FASEB J. *6*, 3338–3344.

Cajigas, I.J., Tushev, G., Will, T.J., tom Dieck, S., Fuerst, N., and Schuman, E.M. (2012). The local transcriptome in the synaptic neuropil revealed by deep sequencing and high-resolution imaging. Neuron *74*, 453–466.

Chen, C.J., Liao, S.L., and Kuo, J.S. (2000). Gliotoxic action of glutamate on cultured astrocytes. J. Neurochem. *75*, 1557–1565.

Chen, Y.W., Song, S., Weng, R., Verma, P., Kugler, J.M., Buescher, M., Rouam, S., and Cohen, S.M. (2014). Systematic study of Drosophila microRNA functions using a collection of targeted knockout mutations. Dev. Cell *31*, 784–800.

Choi, D.W. (1988). Glutamate neurotoxicity and diseases of the nervous system. Neuron *1*, 623–634.

Cornell-Bell, A.H., Finkbeiner, S.M., Cooper, M.S., and Smith, S.J. (1990). Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. Science *247*, 470–473.

David, J.C., Yamada, K.A., Bagwe, M.R., and Goldberg, M.P. (1996). AMPA receptor activation is rapidly toxic to cortical astrocytes when desensitization is blocked. J. Neurosci. *16*, 200–209.

Doherty, J., Logan, M.A., Taşdemir, O.E., and Freeman, M.R. (2009). Ensheathing glia function as phagocytes in the adult Drosophila brain. J. Neurosci. *29*, 4768–4781.

Gehrke, S., Imai, Y., Sokol, N., and Lu, B. (2010). Pathogenic LRRK2 negatively regulates microRNA-mediated translational repression. Nature *466*, 637–641.

Gerstner, J.R., Vanderheyden, W.M., LaVaute, T., Westmark, C.J., Rouhana, L., Pack, A.I., Wickens, M., and Landry, C.F. (2012). Time of day regulates subcellular trafficking, tripartite synaptic localization, and polyadenylation of the astrocytic Fabp7 mRNA. J. Neurosci. *32*, 1383–1394.

Harraz, M.M., Eacker, S.M., Wang, X., Dawson, T.M., and Dawson, V.L. (2012). MicroRNA-223 is neuroprotective by targeting glutamate receptors. Proc. Natl. Acad. Sci. U S A *109*, 18962–18967.

Hilgers, V., Bushati, N., and Cohen, S.M. (2010). Drosophila microRNAs 263a/b confer robustness during development by protecting nascent sense organs from apoptosis. PLoS Biol. *8*, e1000396.

Hollmann, M., Hartley, M., and Heinemann, S. (1991). Ca^{2+} permeability of KA-AMPA–gated glutamate receptor channels depends on subunit composition. Science *252*, 851–853.

Karr, J., Vagin, V., Chen, K., Ganesan, S., Olenkina, O., Gvozdev, V., and Featherstone, D.E. (2009). Regulation of glutamate receptor subunit availability by microRNAs. J. Cell Biol. *185*, 685–697.

Karres, J.S., Hilgers, V., Carrera, I., Treisman, J., and Cohen, S.M. (2007). The conserved microRNA miR-8 tunes atrophin levels to prevent neurodegeneration in Drosophila. Cell *131*, 136–145.

Kim, J., Inoue, K., Ishii, J., Vanti, W.B., Voronov, S.V., Murchison, E., Hannon, G., and Abeliovich, A. (2007). A microRNA feedback circuit in midbrain dopamine neurons. Science *317*, 1220–1224.

Kolodziejczyk, K., Saab, A.S., Nave, K.A., and Attwell, D. (2010). Why do oligodendrocyte lineage cells express glutamate receptors? F1000 Biol. Rep. *2*, 57.

Kouri, F.M., Hurley, L.A., Daniel, W.L., Day, E.S., Hua, Y., Hao, L., Peng, C.Y., Merkel, T.J., Queisser, M.A., Ritner, C., et al. (2015). miR-182 integrates apoptosis, growth, and differentiation programs in glioblastoma. Genes Dev. *29*, 732–745.

Lalo, U., Pankratov, Y., Kirchhoff, F., North, R.A., and Verkhratsky, A. (2006). NMDA receptors mediate neuron-to-glia signaling in mouse cortical astrocytes. J. Neurosci. *26*, 2673–2683.

Letellier, M., Elramah, S., Mondin, M., Soula, A., Penn, A., Choquet, D., Landry, M., Thoumine, O., and Favereaux, A. (2014). miR-92a regulates expression of synaptic GluA1-containing AMPA receptors during homeostatic scaling. Nat. Neurosci. *17*, 1040–1042.

Liu, N., Landreh, M., Cao, K., Abe, M., Hendriks, G.J., Kennerdell, J.R., Zhu, Y., Wang, L.S., and Bonini, N.M. (2012). The microRNA miR-34 modulates ageing and neurodegeneration in Drosophila. Nature *482*, 519–523.

López-Bayghen, E., Espinoza-Rojo, M., and Ortega, A. (2003). Glutamate down-regulates GLAST expression through AMPA receptors in Bergmann glial cells. Brain Res. Mol. Brain Res. *115*, 1–9.

McDonald, J.W., Althomsons, S.P., Hyrc, K.L., Choi, D.W., and Goldberg, M.P. (1998). Oligodendrocytes from forebrain are highly vulnerable to AMPA/kainate receptor-mediated excitotoxicity. Nat. Med. *4*, 291–297.

Miller, M.R., Robinson, K.J., Cleary, M.D., and Doe, C.Q. (2009). TU-tagging: cell type-specific RNA isolation from intact complex tissues. Nat. Methods *6*, 439–441.

Mothet, J.P., Pollegioni, L., Ouanounou, G., Martineau, M., Fossier, P., and Baux, G. (2005). Glutamate receptor activation triggers a calcium-dependent and SNARE protein-dependent release of the gliotransmitter D-serine. Proc. Natl. Acad. Sci. U S A *102*, 5606–5611.

Muthukumar, A.K., Stork, T., and Freeman, M.R. (2014). Activity-dependent regulation of astrocyte GAT levels during synaptogenesis. Nat. Neurosci. *17*, 1340–1350.

Palygin, O., Lalo, U., and Pankratov, Y. (2011). Distinct pharmacological and functional properties of NMDA receptors in mouse cortical astrocytes. Br. J. Pharmacol. *163*, 1755–1766.

Parpura, V., Basarsky, T.A., Liu, F., Jeftinija, K., Jeftinija, S., and Haydon, P.G. (1994). Glutamate-mediated astrocyte-neuron signalling. Nature *369*, 744–747.

Peng, P.L., Zhong, X., Tu, W., Soundarapandian, M.M., Molner, P., Zhu, D., Lau, L., Liu, S., Liu, F., and Lu, Y. (2006). ADAR2-dependent RNA editing of AMPA receptor subunit GluR2 determines vulnerability of neurons in forebrain ischemia. Neuron *49*, 719–733.

Porter, J.T., and McCarthy, K.D. (1997). Astrocytic neurotransmitter receptors in situ and in vivo. Prog. Neurobiol. *51*, 439–455.

Pulver, S.R., Pashkovski, S.L., Hornstein, N.J., Garrity, P.A., and Griffith, L.C. (2009). Temporal dynamics of neuronal activation by channelrhodopsin-2 and TRPA1 determine behavioral output in Drosophila larvae. J. Neurophysiol. *101*, 3075–3088.

Qin, G., Schwarz, T., Kittel, R.J., Schmid, A., Rasse, T.M., Kappei, D., Ponimaskin, E., Heckmann, M., and Sigrist, S.J. (2005). Four different subunits are essential for expressing the synaptic glutamate receptor at neuromuscular junctions of Drosophila. J. Neurosci. *25*, 3209–3218.

Randall, R.D., and Thayer, S.A. (1992). Glutamate-induced calcium transient triggers delayed calcium overload and neurotoxicity in rat hippocampal neurons. J. Neurosci. *12*, 1882–1895.

Robert, A., Hyde, R., Hughes, T.E., and Howe, J.R. (2002). The expression of dominant-negative subunits selectively suppresses neuronal AMPA and kainate receptors. Neuroscience *115*, 1199–1210.

Rothstein, J.D., Dykes-Hoberg, M., Pardo, C.A., Bristol, L.A., Jin, L., Kuncl, R.W., Kanai, Y., Hediger, M.A., Wang, Y., Schielke, J.P., and Welty, D.F. (1996). Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. Neuron *16*, 675–686.

Saab, A.S., Neumeyer, A., Jahn, H.M., Cupido, A., Šimek, A.A., Boele, H.J., Scheller, A., Le Meur, K., Götz, M., Monyer, H., et al. (2012). Bergmann glial AMPA receptors are required for fine motor coordination. Science *337*, 749–753.

Saba, R., Störchel, P.H., Aksoy-Aksel, A., Kepura, F., Lippi, G., Plant, T.D., and Schratt, G.M. (2012). Dopamine-regulated microRNA MiR-181a controls GluA2 surface expression in hippocampal neurons. Mol. Cell. Biol. *32*, 619–632.

Sans, N., Vissel, B., Petralia, R.S., Wang, Y.X., Chang, K., Royle, G.A., Wang, C.Y., O'Gorman, S., Heinemann, S.F., and Wenthold, R.J. (2003). Aberrant formation of glutamate receptor complexes in hippocampal neurons of mice lacking the GluR2 AMPA receptor subunit. J. Neurosci. *23*, 9367–9373.

Sanz-Clemente, A., Nicoll, R.A., and Roche, K.W. (2013). Diversity in NMDA receptor composition: many regulators, many consequences. Neuroscientist *19*, 62–75.

Schratt, G.M., Tuebing, F., Nigh, E.A., Kane, C.G., Sabatini, M.E., Kiebler, M., and Greenberg, M.E. (2006). A brain-specific microRNA regulates dendritic spine development. Nature *439*, 283–289.

Shin, D., Shin, J.Y., McManus, M.T., Ptácek, L.J., and Fu, Y.H. (2009). Dicer ablation in oligodendrocytes provokes neuronal impairment in mice. Ann. Neurol. *66*, 843–857.

Spindler, S.R., Ortiz, I., Fung, S., Takashima, S., and Hartenstein, V. (2009). Drosophila cortex and neuropile glia influence secondary axon tract growth, pathfinding, and fasciculation in the developing larval brain. Dev. Biol. *334*, 355–368.

Tao, J., Wu, H., Lin, Q., Wei, W., Lu, X.H., Cantle, J.P., Ao, Y., Olsen, R.W., Yang, X.W., Mody, I., et al. (2011). Deletion of astroglial Dicer causes non-cell-autonomous neuronal dysfunction and degeneration. J. Neurosci. *31*, 8306–8319.

van Zundert, B., Yoshii, A., and Constantine-Paton, M. (2004). Receptor compartmentalization and trafficking at glutamate synapses: a developmental proposal. Trends Neurosci. *27*, 428–437.

Verkhratsky, A., and Steinhäuser, C. (2000). Ion channels in glial cells. Brain Res. Brain Res. Rev. *32*, 380–412.

Verma, P., Augustine, G.J., Ammar, M.R., Tashiro, A., and Cohen, S.M. (2015). A neuroprotective role for microRNA miR-1000 mediated by limiting glutamate excitotoxicity. Nat. Neurosci. *18*, 379–385.

Warmus, B.A., Sekar, D.R., McCutchen, E., Schellenberg, G.D., Roberts, R.C., McMahon, L.L., and Roberson, E.D. (2014). Tau-mediated NMDA receptor impairment underlies dysfunction of a selectively vulnerable network in a mouse model of frontotemporal dementia. J. Neurosci. *34*, 16482–16495.

Wu, C.L., Xia, S., Fu, T.F., Wang, H., Chen, Y.H., Leong, D., Chiang, A.S., and Tully, T. (2007). Specific requirement of NMDA receptors for long-term memory consolidation in Drosophila ellipsoid body. Nat. Neurosci. *10*, 1578–1586.

Xia, S., Miyashita, T., Fu, T.F., Lin, W.Y., Wu, C.L., Pyzocha, L., Lin, I.R., Saitoe, M., Tully, T., and Chiang, A.S. (2005). NMDA receptors mediate olfactory learning and memory in Drosophila. Curr. Biol. *15*, 603–615.

Yu, B., Qian, T., Wang, Y., Zhou, S., Ding, G., Ding, F., and Gu, X. (2012). miR-182 inhibits Schwann cell proliferation and migration by targeting FGF9 and NTM, respectively at an early stage following sciatic nerve injury. Nucleic Acids Res. *40*, 10356–10365.

Zhang, L.I., and Poo, M.M. (2001). Electrical activity and development of neural circuits. Nat. Neurosci. *4* (*Suppl*), 1207–1214.

Zipfel, G.J., Babcock, D.J., Lee, J.M., and Choi, D.W. (2000). Neuronal apoptosis after CNS injury: the roles of glutamate and calcium. J. Neurotrauma *17*, 857–869.